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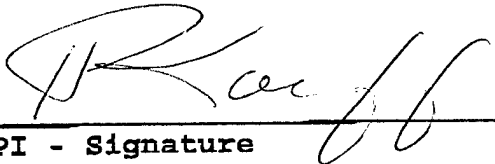
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## TABLE OF CONTENTS

<b>Accession Document</b>	<b>Page 1</b>
<b>Abstract</b>	<b>Page 2</b>
<b>Foreword</b>	<b>Page 3</b>
<b>Table of Contents</b>	<b>Page 4</b>
<b>Introduction</b>	<b>Page 5</b>
<b>Vitamin D 3 analogs and other studies</b>	<b>Page 6</b>
<b>Molecular biology studies</b>	<b>Page 13</b>
<b>Conclusion</b>	<b>Page 13</b>
<b>References</b>	<b>Page 14</b>
<b>Appendices</b>	<b>Page 19</b>

## Introduction

Breast cancer is one of the leading causes of cancer death. 1,25-dihydroxy vitamin D<sub>3</sub> plays an essential role in a variety of cellular mechanisms involved in calcium homeostasis, inhibition of cellular proliferation and induction of cellular differentiation in a variety of cancer cells, such as breast, prostate and colon. 1,25-dihydroxy vitamin D<sub>3</sub> generates cellular responses via gene transcriptions mediated by a 1,25-dihydroxy vitamin D<sub>3</sub> specific nuclear receptor (VDR). We are engaged in a search for vitamin D analogs that will exhibit high potency in cell growth inhibition and cell differentiation activity, without the hypercalcemic side-effects. We are also interested in identifying the molecular alterations associated with breast tumors.

We have completed several *in vitro* and *in vivo* studies of novel vitamin D<sub>3</sub> analogs used alone and in combination against breast cancer. In our prior studies, we synthesized a series of novel 20-cyclopropyl-cholecalciferol vitamin D<sub>3</sub> analogs that were found to be potent inhibitors of clonal growth of MCF-7 breast cancer cells. We examined a new class of Vitamin D analogs that have a novel 5,6-trans motif; the most potent of these analogs affect cell cycle regulatory mechanisms, upregulate expression of cyclin dependent kinase (CDK) inhibitors, inhibit telomerase activity, and induce expression of a novel candidate tumor suppressor gene. We examined the use of vitamin D analogs in combination with All-trans-retinoic acid in human breast tumors in BNX mice and found an additive effect which decreased tumor mass nearly 3-fold with minimal toxicity. Due to the dearth of *in vivo* studies examining the long term effects of Vitamin D analogs, we administered unique analogs for approximately one year to Balb/C mice and performed extensive toxicity analyses which revealed that these compounds were well tolerated with minimal toxicity.

We have shown that a peroxisome proliferator/activated receptor gamma (PPAR $\gamma$ ) (Troglitazone) in combination with All-trans-retinoic acid (ATRA) a ligand for retinoic acid receptor, significantly decreased proliferation and induced differentiation and apoptosis in human breast cancer cells *in vitro* and *in vivo*. Pursuant to these studies, we initiated a clinical trial to examine the safety and efficacy of the synthetic ligand of the peroxisome proliferator/activated receptor gamma (PPAR $\gamma$ ) Troglitazone and All-trans-retinoic acid in patients with metastatic breast cancer. This trial is still ongoing.

We have demonstrated that the organic arsenical, Melarsoprol, has significant activity in breast and prostate cancers. This compound may have efficacy as a novel treatment for breast cancer.

We continue to search for genetic alterations of breast cancer; our studies include examination of a mutation of the p16<sup>INK4A</sup> binding domain of the CDK4 gene and evaluation of the novel tumor suppressor gene DPC4/SMAD4 in diverse types of cancers, including human breast cancer. To better understand the role of cyclin dependent kinase inhibitors in breast cancer, and a variety of other neoplasms were examined for p21<sup>WAF1</sup>, p27<sup>KIP1</sup>, p15<sup>INK4B</sup>, p16<sup>INK4</sup>, p18<sup>INK4C</sup> and 19<sup>INK4</sup> alterations.

We have demonstrated that 1,25(OH)<sub>2</sub>-D<sub>3</sub> (vitamin D<sub>3</sub>) modulates BRCA1 expression in a panel of breast and prostate cancer cell lines and that the extent to which either vitamin D<sub>3</sub> or its analogs modulates BRCA1 is often proportional to their ability to be clonally inhibited. Reducing vitamin D receptor content in one cell line reduces the clonal sensitivity and ability to induce BRCA1. These data suggest that BRCA1 protein expression is an important pathway for controlling cell proliferation in both breast and prostate cells. We speculate that some aspect of Vitamin D<sub>3</sub> signaling, such as co-activator is lost in these cells that selectively reduce transactivation of genes that are critical to controlling cellular proliferation.

## Body

### 1. Vitamin D<sub>3</sub> and Other Studies:

a. The active vitamin D<sub>3</sub> metabolite 1,25-dihydroxyvitamin D<sub>3</sub> (compound C) is an important modulator of cellular proliferation and differentiation in a variety of normal and malignant cells. Most breast cancer cell lines and more than 80% of breast tumors express high affinity intracellular vitamin D receptors (VDR) (1-4). The hypercalcemic effect of vitamin D<sub>3</sub> has limited its clinical utility. Vitamin D<sub>3</sub> analogs have been developed that inhibit cellular proliferation and induce differentiation without the attendant hypercalcemia (6-8). Vitamin D<sub>3</sub> analogs and Taxol are able to inhibit the *in vitro* growth of a variety of malignant cells including breast cancer cells. These compounds decrease growth by different mechanisms and have non-overlapping toxicities. We examined three vitamin D<sub>3</sub> analogs to inhibit MCF-7 human mammary cancers in BNX triple immunodeficient mice, either alone or in combination.

One of the exciting new analogs identified to date is 1,25-(OH)<sub>2</sub>-16-ene-23-yne-26,27-F<sub>6</sub>-19-nor-D<sub>3</sub> (compound LH). This potent analog reduced the development of breast cancer in nitroso-N-methylurea-treated rats (10). Furthermore, dose-response studies showed that it was one of the most potent vitamin D<sub>3</sub> analog in suppressing clonogenic cancer growth, being able to suppress at 10<sup>-11</sup> M greater than 50 % clonal proliferation of the MCF-7 and SK-BR-3 breast cancer cells. The analog increased the proportion of cancer cells in G<sub>0</sub>/G<sub>1</sub> phases and decreased those in the S phase of the cell cycle (9). Pulse-exposure studies showed that three day exposure to LH (10<sup>-7</sup> M) in liquid culture was able to achieve a 50% inhibition of MCF-7 clonal growth in soft-agar in the absence of analog suggesting that inhibition of growth mediated by Compound LH is irreversible (9). Further studies have found that the cyclin dependent kinase inhibitor known as p27<sup>Kip1</sup> is induced at high levels by Compound LH in the MCF-7 and SK-BR-3 breast cancer cells (9).

The analog 24a,26a,27a-trihomo-22,24-diene-1,25(OH)<sub>2</sub>D<sub>3</sub> (Compound EB1089) has a wide spectrum of anti-cancer activities *in vitro* including breast cancer cells (11-14); the analog is between 7-50-fold more potent than the parental 1,25(OH)<sub>2</sub>D<sub>3</sub> (Compound C) *in vitro* against cancer cells. It appears to have similar effects on serum calcium levels as compared to 1,25(OH)<sub>2</sub>D<sub>3</sub> (11). A Phase I study in patients with advance breast cancer showed that EB1089 can be given with predictable effects on cancer metabolism (14). A

Phase II trial of this novel vitamin D<sub>3</sub> analog is currently underway in patients with breast carcinoma.

The taxanes are an important new class of anticancer agents that exert their cytotoxic effects through a unique mechanism. Paclitaxel (Taxol) stabilizes microtubules and inhibits their depolymerization to free tubulin. It can block mitosis, induce extensive formation of microtubule bundles in cells, and cause multinucleation of cells during interphase (15-17). Taxol, the first taxane in clinical trials, is active against a broad range of cancers that are generally considered to be refractory to conventional chemotherapy. This has led to the regulatory approval of Taxol in many countries for use as palliative therapy of patients with ovarian and breast cancers resistant to chemotherapy. Taxol was discovered as part of a National Cancer Institute program in which extracts of thousands of plants were screened for anticancer activity (15). Taxol-based combinations, especially those with doxorubicin or cisplatin, appear promising for further study (18,19).

The vitamin D<sub>3</sub> compounds and taxol have non-overlapping toxicities. To our knowledge, no one has studied the ability of the combination of a vitamin D<sub>3</sub> analog and Taxol to inhibit growth of human breast cancer in vivo. We report that this combination of therapy is efficacious in inhibiting the growth of MCF-7 tumors in BNX nude mice.

In our studies animals were bilaterally, subcutaneously injected with 10<sup>6</sup> MCF-7 cells/tumor in 0.1 ml Matrigel (Collaborative Biomedical Products, Bedford, MA). Before injection of cells, the animals received 300 rads whole body irradiation. Mice were divided randomly into eight groups of five mice each: Group A: nontreatment (control); Group B: Taxol; Group C: Compound C; Group D: Compound C + Taxol; Group E: Compound LH; Group F: Compound LH + Taxol; Group G: EB1089; and Group H: EB1089 + Taxol.

Vitamin D<sub>3</sub> analogs were administered intraperitoneally every other day at the following doses: Compound C, 0.05 µg/mouse; Compound LH, 0.0125 µg/mouse; and EB1089, 0.05 µg/mouse. The doses were chosen after a series of initial experiments determined the highest dose of the vitamin D<sub>3</sub> that could be given without causing hypercalcemia. Taxol (25 mg/kg/mouse) was administered intraperitoneally once a week. The dose was chosen from the report of Kalechman, K. et.al (19). One day after tumor injections, mice were treated with either vitamin D<sub>3</sub> analogs alone, Taxol alone, or the combination of a vitamin D<sub>3</sub> analog and Taxol. During the experiment, four mice died: one in the Compound C + Taxol group; one in the Compound LH + Taxol group; and two in the EB1089 + Taxol group. The cause of their deaths was unknown.

Tumors were measured every week with vernier calipers. Tumor size index was calculated by the formula:  $a \times b \times c$ , where  $a$  is the length and  $b$  is the width and  $c$  is the height in millimeters. Serum calcium values were measured on days 20 and 68 by atomic absorption spectrophotometry (Perkin-Elmer 560) and a modification of the calcium o-cresolphthalein complexone complexometric reaction (Dupont Analyst Benchtop Chemistry System, Dade International).

At the end of the experiment, animals were killed by CO<sub>2</sub> asphyxiation and tumor weights were measured after careful resection, and blood was also collected from the orbital sinus for chemistry and blood analysis. Chemistries and blood analyses were measured by Dupont Analyst Benchtop Chemistry System, Dade International, Newark, DE and by Serono-Baker 9000 Diff, Biochem Immuno-Systems, Allentown, PA, respectively.

At completion of the trial, all the treatment groups had statistically significantly smaller tumors than the non-treated group. Administration of vitamin D<sub>3</sub> analogs alone remarkably suppressed the growth of the tumors. The most potent single agent was EB1089 (Group G). The second in potency was Compound LH (Group E), followed by Compound C. The antitumor effect of vitamin D<sub>3</sub> analogs appeared greater than that of Taxol and the enhanced activity was observed when vitamin D<sub>3</sub> analog and Taxol were administered together. In each case, the combination of a vitamin D<sub>3</sub> compound and Taxol suppressed tumor growth greater than either alone.

The results were similar when the effect of vitamin D<sub>3</sub> analogs and Taxol were evaluated by tumor weight at the conclusion of the study (Figure 1). Again, EB1089 was the most potent single agent, and the combination of a vitamin D<sub>3</sub> compound and Taxol was more

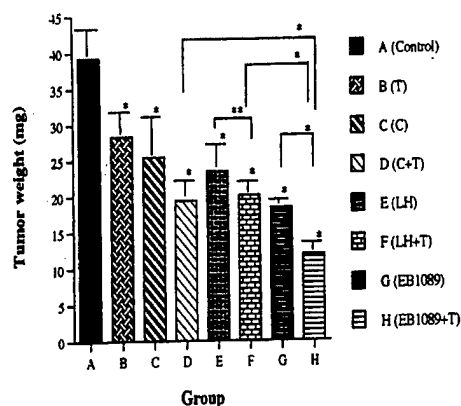


Figure 1. Effect of vitamin D<sub>3</sub> analogs and Taxol, either alone or in combination, on the weight of MCF-7 tumors in BNX nude mice. MCF-7 breast cancer tumors were established by subcutaneous injection of the cells. Vitamin D<sub>3</sub> analogs were administered intraperitoneally (i.p.) every other day except Saturday and Sunday. Taxol was administered i.p. once a week. After nine weeks of injections, tumors were dissected and weighed. Results represent means  $\pm$  SD of six to ten tumors: a, significantly ( $p < 0.01$ ) different from Group A. \*, and \*\* represent data that are statistically significant at  $p < 0.01$  and  $p < 0.05$ , respectively, as determined by Mann-Whitney U-test.

potent than either alone. Tumor weights in the combined treatment groups were approximately 30% to 50% of those in the no treatment group. All the treatment groups were statistically different from Group A (control,  $p < 0.01$ ). In addition, Group H (EB1089 + Taxol) was statistically different from Groups D (Compound C + Taxol) and F (Compound LH + Taxol).

The dose of these vitamin D<sub>3</sub> analogs that caused a remarkable inhibition of the size and weight of

breast cancer did not elevate the level of the serum calcium (normal 8.5-10.5 mg/dl). We believe that initial calcium values were lower in all mice including controls than later in the study as a consequence of using two different methods of measurement. In the first measurement, we used atomic absorption spectrometry and in the second we used a modification of the calcium o-cresolphthalein complexone complexometric reaction.

During the study, all mice were weighed once per week (Figure 2). Each of the cohorts gained in weight, but groups D (Compound C + Taxol) and G (EB1089) had statistically lower body weights than the non-treated group. The body weights in these three groups were 82-87% of that in the control Group A. The body weights of the other cohorts were not statistically different compared to the untreated control Group A. In general, each of

the mice looked healthy.

The present data show that the vitamin D<sub>3</sub> analogs and Taxol had potent anti-breast cancer activity *in vivo* without causing hypercalcemia and other major side-effects. Combined treatment of the MCF-7 human breast cancer cells resulted in a stronger inhibition than treatment with either a vitamin D<sub>3</sub> compound or Taxol alone.

Antiestrogen therapy is the pivotal endocrine therapy of breast cancer (20). However, breast cancer patients whose tumors do not express estrogen receptors constitute 30-40% of breast cancer patients (5); and they have a significantly worse prognosis than those with estrogen receptors (21). Furthermore, resistance to antiestrogen therapy frequently occurs (22); in these situations, treatment with vitamin D<sub>3</sub> might be useful.

The 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs can inhibit tumor growth by a variety of mechanisms, including regulation of angiogenesis, apoptosis, tumor invasiveness and G<sub>0</sub>/G<sub>1</sub> cell cycle arrest as a result in part of the enhanced expression of the cyclin dependent kinase inhibitors known as p21<sup>WAF1</sup> and p27<sup>Kip1</sup> (23-27). Despite promising antitumor activity of 1,25(OH)<sub>2</sub>D<sub>3</sub> *in vitro*, its calcemic toxicity *in vivo* limits the doses that can be given. The vitamin D<sub>3</sub> analogs LH and EB1089 have almost the same growth inhibitory action as Compound C, but 50-100-fold lower concentrations of these analogs were required for this anticancer activity. In contrast, *in vivo* studies have shown that the calcemic activity of the EB1089 analog was lower than 1,25(OH)<sub>2</sub>D<sub>3</sub> (11,28-30). The Compound LH had slightly higher calcemic activity than 1,25(OH)<sub>2</sub>D<sub>3</sub> (11). Our data show that the growth inhibitory action of the three vitamin D<sub>3</sub> compounds was statistically greater than that of the non-treatment group. Moreover, each of these cohorts had inhibition of tumor growth without hypercalcemia.

Taxol is one of the most important new cytotoxic agents to be introduced for the management of breast cancer in several years (15-19). Combinations of Taxol with various cytotoxic agents are being actively explored (31-34). As expected, the present data show that taxol has an anti-breast cancer effect *in vivo*. The combination of one of the vitamin D<sub>3</sub> compounds with Taxol remarkably suppressed the growth of human breast cancer cells *in vivo* (Figure 1). This was shown most impressively when examining the tumor weights at the conclusion of the study, which decreased 70% in the mice that received the combination of EB1089 with Taxol as compared to that in the diluent-treated control group.

Chemotherapy of many stages of breast cancer is still based on the combined use of three major classes of anticancer drugs: alkylating agents, antimetabolites, and anthracycline antibiotics. Nevertheless, these combined chemotherapies are associated with overlapping toxicities and are not completely effective. Therefore, combinations of different forms of therapy including biologic modifiers such as vitamin D<sub>3</sub> analogs combined with Taxol, as well as antiestrogens, and retinoids may be worthwhile.

Taxol exerts its cytotoxic effects through a unique mechanism of microtubules

stabilization resulting in blockade of mitosis (16,17,35). The vitamin D<sub>3</sub> compounds are lipid soluble and freely enter the cell. They bind and activate the vitamin D<sub>3</sub> receptors, allowing efficient interaction with vitamin D response elements thus modulating the expression of various genes. Despite intense research, the exact mode of action by which vitamin 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs inhibit cancer cells growth remains largely unknown (36). They can lead to cell cycle arrest with elevation in levels of p21<sup>WAF1</sup> and p27<sup>KIP1</sup> cyclin dependent inhibitors. Taken together, the vitamin D<sub>3</sub> compounds and taxol probably inhibit proliferation of cancer cells including those of the breast by different mechanisms. Furthermore, the toxicities of the two therapies are clearly different, with the vitamin D<sub>3</sub> compounds potentially producing hypercalcemia and the taxols having the ability to cause hematopoietic cytopenias.

b. The prevention of cancer (chemoprevention) is clearly more cost effective than the treatment of an established cancer and may represent the best approach to this disease. We (and others) have shown that activation of the peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) by thiazolidinediones, including the synthetic ligand Troglitazone (TGZ), inhibits cultured breast cancer cell lines (37,38). All-trans-retinoic acid (ATRA) also inhibits *in vitro* proliferation of breast cancer cells and the combination of TGZ and ATRA caused significant apoptosis of MCF-7 breast tumors in mouse models without significant toxicity (38).

Retinoids (RA) mediate their activity via the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). PPAR $\gamma$  is a member of the nuclear hormone receptor superfamily that includes retinoic acid receptors (RAR and RXR) and thyroid hormone receptors. PPAR $\gamma$  heterodimerizes with RXR and binds to DNA, resulting in expression of genes associated with many aspects of differentiation, cellular development and general physiology (39,40). The PPAR $\gamma$  ligand TGZ is useful for the treatment of Type 2 diabetes and has been used to treat over 1 million individuals with this disease. These agents may enhance differentiation of adipocytes and thus may be associated with upregulation of their glucose pumps, however, the exact mechanism of action is unclear. All-trans-retinoic acid, an RAR specific ligand, selectively inhibits growth of ER-positive breast cancer cells (41,42) and is also effective in preventing mammary carcinogenesis in rodents (43). The lack of toxicity for most individuals receiving TGZ for adult onset diabetes, as well as the lack of adverse effects of several RXR ligands including 9-cis retinoic acid, makes the combination of TGZ and a RXR analog an attractive combination for *in vivo* chemoprevention trials. Furthermore, there are several new thiazolidinediones now available; these compounds do not appear to have the idiosyncratic liver toxicity that occurs rarely with administration of TGZ.

The murine mammary gland organ culture system effectively evaluates the effects of potential chemopreventive agents to inhibit the induction of preneoplastic lesions (44-47). Mammary glands of BALB-C mice are placed in organ cultures containing a variety of growth proliferative factors and hormones and are treated with the carcinogen 7,12-dimethylbenzine[a]anthracene (DMBA) to induce preneoplastic lesions. The mammary epithelial cells isolated from these lesions, are placed into synergistic hosts and

subsequently develop adenocarcinomas. Using this technique over 150 different chemopreventive agents have been tested. This assay is highly reproducible and provides a good correlation with the efficacy of a chemopreventive agent in both *in vitro* and *in vivo* models. We have used this model system to analyze the efficiency of TGZ and/or a retinoid to prevent the formation of DMBA-induced mammary lesions (MAL) in a murine mammary gland organ culture model.

In our studies we found that combining a PPAR $\gamma$  with a ligand specific for RXR (LG10069) enhanced the suppression of development of mammary lesions. Previous studies have shown that simultaneous activation of both receptors can result in synergistic activity in several assays of cultured cells as well as augmented *in vivo* anti-diabetic activity (48). Furthermore, we have previously shown that a PPAR $\gamma$  ligand and a RXR ligand can have enhanced antiproliferative effects against both breast and prostate cancer cells (38,49). Previous studies have shown that a RXR-specific agonist (LG10069) had chemopreventive activity against chemically induced rat mammary tumors (50). However, such activity for LG10068 has not been reported.

We examined the effect of TGZ combined with a RXR ligand (LG10068). The RXR ligand ( $10^{-7}$  -  $10^{-8}$  M) was unable to inhibit DMBA-induced mammary lesions (figure 1A shows a DMBA-induced mammary lesion), and TGZ ( $10^{-6}$ ) in this series of experiments inhibited mammary lesions only by approximately 14% (Table 1).

**Table 1: Effects of Troglitazone and/or ATRA on the Development of DMBA-Induced Mammary Lesions**

Group	Treatment	Glands with Lesions	Incidence	Inhibition	Chi Square  p=<
			of Lesions  %	of Lesions  %	
1	DMBA	26/30	87		
2	TROG ( $10^{-6}$ M)	12/20	60	31	0.06
3	TROG ( $10^{-5}$ M)	7/20	35	60	0.005
4	ATHA ( $10^{-8}$ M)	5/10	50	43	0.05
5	ATRA ( $10^{-7}$ M)	4/10	40	56	0.01
6	ATRA ( $10^{-6}$ M)	2/10	20	77	0.003
7	TROG $10^{-6}$ M, ATRA ( $10^{-8}$ M)	4/10	40	54	0.01
8	TROG $10^{-5}$ M, ATRA ( $10^{-8}$ M)	3/10	30	66	0.005
9	TROG $10^{-5}$ M, ATRA ( $10^{-7}$ M)	0/10	0	100	0.001
10	TROG $10^{-6}$ M, ATRA ( $10^{-6}$ M)	2/10	20	77	0.003
11	TROG $10^{-5}$ M ATRA $10^{-6}$ M	1/10	10	89	0.001

Trog, troglitazone; ATRA, all-trans retinoic acid; DMBA, 7, 12-dimethyl-benz[a]anthracene.

However, when the two were combined, the percent inhibition of development of breasts with abnormalities was 85%, showing that the two ligands together were clearly more effective than either alone (Table 2).

**Table 2** Effects of Troglitazone and/or RXR Analog on the Development of DMBA-Induced Mammary Lesions.

Group	Treatment	Glands with Lesions	Incidence of Lesions (%)	(%) Inhibition of Lesions	Chi Square
					P=<
1	DMBA	7/10	70		
2	TROG ( $10^{-6}$ M)	6/10	60	14	NS
3	TROG ( $10^{-5}$ M)	1/10	10	85	0.02
4	RXR ( $10^{-7}$ M)	6/9	67	5	NS
5	RXR ( $10^{-8}$ M)	8/10	80	0	NS
6	TROG ( $10^{-6}$ M)+ RXR ( $10^{-8}$ M)	1/10	100	85	0.02
7	TROG ( $10^{-5}$ M)+ RXR ( $10^{-8}$ M)	0/10	0	100	0.01
8	TROG ( $10^{-5}$ M) (0-4 days)	2/10	20	71	0.024
9	TROG ( $10^{-5}$ M) (4- 10 days)	2/10	20	71	0.024

RXR, LG 10068 ligand (retinoid that selectively binds RXR $\alpha$ ), Trog, troglitazone

The PPAR $\gamma$  heterodimerizes with RXR, and each can simultaneously bind to their ligand resulting in enhanced activity of this activated receptor complex. These results are shown in a representative photograph of the gland (figure 1B). Additional studies showed that TGZ probably inhibited both the initiation as well as the progression of the DMBA-induced lesions. Further studies are required to determine the target genes associated with this anti-cancer activity.



This is the first report showing the possibility of a PPAR $\gamma$  ligand having chemopreventive activity. Troglitazone is a relatively non-toxic compound at a wide range of concentrations, but it is a potent inhibitor of the development of preneoplastic lesions of the mammary gland in organ culture. Also, a RXR- or RAR-selective retinoid appears to enhance this chemopreventive activity; thus, the combination of a thiazolidinedione with a retinoid, such as either

**Figure 1**

ATRA or LG10068, may be a good candidate for an *in vivo* breast cancer chemoprevention study. Individuals at high risk for developing breast cancer can be identified due to the recent advances in genetics and the epidemiology of breast cancer. It is these individuals who may receive the most benefit from a chemoprevention regimen containing a PPAR $\gamma$  ligand combined

with a retinoid.

Mehta RG, Williamson E and Koeffler HP. PPAR $\gamma$  Ligand and Retinoids Prevent Preneoplastic Mammary Lesions. **In Press JNCI** (publication date 3/1/00)

## **2. Molecular Alterations in Breast Cancer:**

To continue our work to identify genes that are involved in the tumorigenesis of breast cancer, PCR-selected cDNA subtraction was utilized to construct a breast cancer subtracted library. Differential screening of the library isolated the growth factor inducible immediate-early gene Cyr61, a secreted, cysteine-rich, heparin binding protein that promotes endothelial cell adhesion, migration and neovascularization. Northern analysis revealed that Cyr61 expression correlated with invasiveness and tumorigenicity in breast tumor cells. Cyr61 is expressed highly in invasive, ER negative breast cancer cell lines MDA-MB-231, SK-BR-3 and MDA-MB-157, at very low level in less tumorigenic, ER positive cells MCF7, T47D and BT-20, and barely detectable in normal breast cell MCF12A. Significantly, high expression of Cyr61 was found in about 40% breast tumor biopsies tested. Interestingly, expression of Cyr61 in breast cells is modulated by both estrogen and antiestrogen in a time- and dose-dependent manner. Expression of Cyr61 increased 8-12 fold in MCF12A and 3-5 fold in MCF7 after 48 hr estrogen treatment. The induction of Cyr61 was blocked by tamoxifen, an estrogen receptor inhibitor. These results suggest that Cyr61 may play a role in the progression of breast cancer and may be involved in estrogen-mediated tumor development.

Xie D, Nakachi K, Higashi Y, Sakashita A, Miller C, Koeffler HP. Cyr61, an angiogenic inducer, is overexpressed and estrogen inducible in breast cancer. AACR Abstract submission

## **Conclusions:**

Our data demonstrate that the combination of a vitamin D<sub>3</sub> analog and Taxol markedly inhibited the growth of human breast cancer cells (MCF-7) *in vivo* without causing either hypercalcemia, hematopoietic cytopenias or other major side-effects. This combination has the potential for treatment of breast cancer patients, especially in the adjuvant setting.

Chemoprevention of breast cancer is an active area of basic science and clinical investigation. Our work is the first report showing the possibility of a PPAR $\gamma$  ligand having chemopreventive properties. Furthermore, an RXR-selective ligand appears to enhance this activity. Because both of these drugs possess relatively minor toxicity profiles, this combination may be a good candidate for an *in vivo* breast cancer chemoprevention study.

Cyr61 has previously been identified in other tissue types and has been associated with angiogenesis and metastasis. We have found that selected breast cancer cell lines as well as an array of fresh breast cancers have prominent expression of this gene. Interestingly, normal breast tissue did not express this gene at all. However, the gene was activated when the normal breast cells were exposed to estrogen, and expression was blocked when exposed to

an estrogen receptor inhibitor (tamoxifen). Our studies of Cyr61 may have important implications for our understanding of the progression of breast cancer.

## STATEMENT OF WORK

Specific Aim 1: We continue to pursue our studies of novel vitamin D<sub>3</sub> analogs as single agents or in combination for their ability to inhibit clonal proliferation, and induce apoptosis and differentiation of breast cancer cells.

Specific Aim 2: We have studied the effects of vitamin D<sub>3</sub> analogs in our *in vivo* models.

Specific Aim 3: We have identified new genes associated with the tumorigenesis of breast cancer.

Specific Aim 4: We continue our collaboration with Dr. Daniela Sanitoli in examining the effects of TALL-104 cells *in vitro* and *in vivo* in breast cancer cells.

## References:

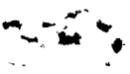
1. Berger, U., Wilson, P., McClelland, R.A., Colston, K., Haussler, M.R., Pike, J.W., and Coombes, R.C. Immunocyto-chemical detection of 1,25-dihydroxyvitamin D receptors in breast cancer. *Cancer Res.*, 47: 6793-6799, 1987.
2. Buras, R.R., Schumaker, L.M., Davoodi, f., Brenner, R.V., Shabahang, M., Nauta, R.J., Evans, S. R. Vitamin D receptors in breast cancer cells. *Breast Cancer Research and Treatment* 31:191-202, 1994.
3. Frealce, H., Abayasakera, G., Iwasaki, J., Marcocci, C., MacIntyre, I., McClelland, R., Skilton, R., Easton, D., and Coombes, R.C. Measurement of 1,25-dihydroxyvitamin D<sub>3</sub> receptors in breast cancer and their relationship to biochemical and clinical indices. *Cancer Res.*, 44: 1677-1681, 1987.
4. Eisman, J., Suva, L., Sher, E., Pierce, P., Funder, J., and Martin, T.J., 1,25-dihydroxyvitamin D<sub>3</sub> receptor in human breast cancer. *Cancer Res.* 41: 5121-5124, 1981.
5. Colston, K.W., Berger, U., and Coombes, R.C. Possible role for vitamin D in controlling breast cancer cells proliferation. *Lancet.* 28: 188-191, 1989.
6. Colston, K.W., Chander, S.K., Mackay, A.G., and Coombes, R.C. Effects of synthetic vitamin D analogues on breast cancer cell proliferation *in vivo* and *in vitro*. *Biochem. Pharmacol.*, 44: 693-702, 1992.
7. Abe, J., Nakano, T., Nishi, Y., Matsumoto, T., Ogata, E., and Ikeda, K. A novel vitamin D<sub>3</sub> analog, 22-Oxa-1,25-dihydroxyvitamin D<sub>3</sub>, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. *Endocrinology*, 129: 832-837, 1991.
8. Bower, M., Colston, K.W., Stein, R.C., Hedley, A., Gazet, J.C., Ford, H.T., and Coombes, R.C. Topical calcipotriol treatment in advanced breast cancer. *Lancet*, 337: 701- 702, 1991.
9. Koike M, Elstener E, Campbell, M.J., Asou, H., Uskokovic, M., Tsuruoka, N., and

- Koeffler, H.P. 19-nor-Hexafluoride analogue of vitamin D<sub>3</sub>: A novel class of potent inhibitors of proliferation of human breast cell lines. *Cancer Res.* 57:4545-4550, 1997
10. Anzano, M.A., Smith, J.M, Uskokovic, M.R., Peer, C.W., Mullen, L.T., Letterio, J.J., et al. 1- $\alpha$ ,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (Ro24-5531), a new deltanoid (vitamin D analogue) for prevention of breast cancer in the rat. *Cancer Res* 54:1653-1656, 1994.
  11. Pakkala, S., de Vos, S., Elstner, E., Rude, B., Uskokovic, M., Binderup, L., and Koeffler, H.P. Vitamin D<sub>3</sub> analogs: effect on leukemic clonal growth and differentiation, and serum calcium. *Leuk. Res.*, 19:65-71, 1995.
  12. Love-Schimenti, C.D., Gibson, D.F., Ratnam, A.V., and Bikle, D.D. Antiestrogen potentiation of antiproliferative effects of vitamin D<sub>3</sub> analogues in breast cancer cells. *Cancer Res.*, 56:2789-2794, 1996.
  13. Zugmaier, G., Jager, R., Grage, B., Gottardis, M.M., Havemann, K., and Knabbe, C. Growth-inhibitory effects of vitamin D analogues and retinoids on human pancreatic cancer cells. *Br. J. Cancer*, 73:1341-1346, 1996.
  14. Colston, K.W., Mackay, A.G., James, S.Y., Binderup, L., Chander, S., and Coombes, C. EB1089: A new vitamin D analogue that inhibits the growth of breast cancer cells in vivo and in vitro. *Biochem. Pharmacol.*, 44:2273-2280, 1992.
  15. Rowinsky, E.K., and Donehower, R.C. Paclitaxel (Taxol). *N.Engl.J.Med.*, 332: 1004-1014, 1995.
  16. Schiff, P.B., Gant, J., and Horwitz, S.B. Promotion of microtubule assembly in vitro by Taxol. *Nature*, 277: 655, 1979.
  17. Schiff, P.B., and S.B. Horwitz. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc.Natl.Acad. Sci.,USA*, 77: 1561-1565, 1980.
  18. O'Shaughnessy, J.A., Fisherman, J.S., and Cowan, K.H. Combination paclitaxel (Taxol) and doxorubicin therapy for metastatic breast cancer. *Semin.Oncol.*, 21: 19-23, 1994 (suppl 8).
  19. Kalechman, Y., Shani, A., Dovrat, S., Whisnant, J.K., Mettiger, K., Albeck, M., and Sredni, B. The antitumoral effect of the immunomodulator AS101 and paclitaxel (Taxol) in a murine model of lung adenocarcinoma. *J. Immunol.*, 156:1101-1109, 1996.
  20. Early Breast Cancer Trialist's Collaborative Group: Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 Randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet*, 339: 1-15, 71-85, 1992.
  21. Santen, R.J., Manni, A., Harvey, H., and Redmond, C. Endocrine treatment of breast cancer in women. *Endocr. Rev.*, 11:221-265, 1990.
  22. Dorssers, L.C.J., Van Agthoven, T., Dekker, A., Van Agthoven, T.L.A., and Kok, E.M. Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of brca-1, a common integration site. *Mol.Endocrinol.*, 7: 870-878, 1993.
  23. Oikawa, T., Hirotsu, K., Ogasawara, H., Katayama, T., Nakamura, O., Iwaguchi, T., and Hiragun, A. Inhibition of angiogenesis by vitamin D<sub>3</sub> analogs.

- Eur.J.Pharmacol., 178: 247-250, 1990.
24. Elstner, E., Linker-Israeli, M., Umiel, T., Le, J., Grillier, I., Said, J., Shintaku, I.P., Krajewski, S., Reed, J.C., Binderup, L., and Koeffler, P.H. Combination of a potent 20-epi-vitamin D<sub>3</sub> analog(KH1060) with 9-cis-retinoic acid irreversibly inhibits clonal growth, decreases bcl-2 expression and induces apoptosis in HL-60 leukemia cells. *Cancer Res.*, 56: 3570-3576, 1996.
  25. Hansen, C.M., and Binderup, L. Effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> and some selected analogues on invasive potential of human carcinoma cells in vitro. Abstract of 9th Workshop on vitamin D, Orlando, Florida, p.20, 1994.
  26. Wang, Q.M., Jones, J.B., and Studzinski, G.P. Cyclin-dependent kinase inhibitor p27 as a mediator of the G<sub>1</sub>-S phase block induced by 1,25-dihydroxyvitamin D<sub>3</sub> in HL-60 cells. *Cancer Res.*, 56: 264-267, 1996.
  27. Munker, R., Kobayashi, T., Elstner, E., Norman, A.W., Uskokovic, N., Zhang, W., Michael, A., and Koeffler, H.P. A new series of vitamin D analogs is highly active for clonal inhibition, differentiation, and induction of WAF1 in myeloid leukemia. *Blood.*, 88: 2201-2209, 1996.
  28. Wijngaarden, T.V.-v., Pols, H.A.P., Buurman, C.J., van den Bermd, G.J.C.M., Dorssers, L.C.J., Birkenhäger, L.C., and van Leeuwen, J.P.T.M. Inhibition of breast cancer cell growth by combined treatment with vitamin D<sub>3</sub> analogues and tamoxifen. *Cancer Res.*, 54: 5711-5717, 1994.
  29. Binderup, L., Latini, S., Binderup, E., Bretting, C., Calverley, M.J., and Hansen, K. 20-epi-vitamin D<sub>3</sub> analogues: a novel class of potent regulators of cell growth and immune responses. *Biochem. Pharmacol.*, 42:1569-1575, 1991.
  30. Binderup, E., Calverley, M.J., and Binderup, L. Synthesis and biological activity of 1 $\alpha$ -hydroxylated vitamin D analogues with polyunsaturated side chains. In: Norman, A.W., Bouillon, R., and Thomasset, M(eds.), *Vitamin D gene regulation, structure-function analysis and clinical application*, pp.192-193. Berlin, Germany: Walter de Gruyter, 1991.
  31. Harn, S.M., Liebmann, J.E., Cook, John., Fisher, J., Goldspiel, B., Venzon, D., Mitchell, J.B., and Kaufman, D. Taxol in combination with doxorubicin or etoposide. *Cancer*, 72: 2705-2711, 1993.
  32. Capri, G., Tarenzi, E., Fulfarò, F., and Gianni, L. The role of taxanes in the treatment of breast cancer. *Semin. Oncol.*, 23: 68--75, 1996(Supple 2).
  33. Tolcher, A.W. Paclitaxel couplets with cyclophosphamide or cisplatin in metastatic breast cancer. *Semin.Oncol.*, 23:37-43, 1996(Suppl 1).
  34. Seidman, A.D., Reichman, B.S., Crown, J.P.A., Yao, T.-J., Carrie, V., Hanks, T.B., Hudis, C.A., Gilewski, T.A., Baselga, J., Forsythe, P., Lepore, J., Marks, L., Fain, K., Souhrada, M., Onetto, N., Arbut, S., and Norton, L. Paclitaxel as second and subsequent therapy for metastatic breast cancer: activity independent of prior anthracycline response. *J.Clin.Oncol.*, 13: 1152-1159, 1995.
  35. Rowinsky, E.K. and Donehower, R.C: Paclitaxel (Taxol). *N Engl J Med* 332:1004-1014, 1995.
  36. Campbell MJ, and Koeffler, H.P. Toward Therapeutic Intervention of Cancer by Vitamin D Compounds. *J of Nat Cancer Inst.* 89(3)182-185, 1997.

37. Mueller E, Sarraf P, Tontonoz P, Evans RM, Martin KJ, Zhang M, et. al. Terminal differentiation of human breast cancer through PPAR $\gamma$ . *Molecular Cell*, 1:465-70, 1998.
38. Elstner E, Miller C, Koshizuka K, Williamson EA, Park D, Asou H, et. al. Ligands for peroxisome proliferator activated receptor  $\gamma$  and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. *Proc. Natl. Acad. Sci USA*, 95:8806-11, 1998.
39. Chawla A, Schwarz EJ, Dimaculangan DD and Lazar MA. Peroxisome proliferator activated receptor PPAR  $\gamma$ : adipose-predominant expression and induction early in adipocyte differentiation. *Endocrinology*, 135:798-800, 1994.
40. Tontonoz P, Singer S, Forman B, Sarraf P, Flecher J, Flecher CDM, et al. Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator activated receptor and the retinoid X receptor. *Proc Natl Acad Sci USA*, 94:2337-241, 1997.
41. Marth C, Mayer I, and Daxenbichler G. Effect of retinoic acid and 4-hydroxytamoxifen on human breast cancer cell lines. *Biochem Pharmacol*, 33:2217-2221, 1984.
42. Van der Burg B, van der Leed BM, Kwakkenbos-Isbrucker L, Salverda S, deLaat S, van der Saag PT. Retinoic acid resistance of estradiol-independent breast cancer cells coincides with diminished retinoic acid receptor function. *Mol Cell Endocrinol*, 91:149-157, 1993.
43. Moon RC, Mehta RG, Rao KVN. The retinoids: Biology. In: Sporn MB, Roberts AB, and Goodman DS, editors. *Chemistry and Medicine*. New York (NY): Raven, p. 573-595, 1994.
44. Mehta RG, Liu J, Constantinou A, Thomas CF, Hawthorn M, You M, et al. Cancer chemopreventive activity of brassinin, a phytoalexin from cabbage. *Carcinogenesis*, 16:399-404, 1995.
45. Mehta RG, Steele V, Kelloff GJ, Moon C. Influence of thiols and inhibitors of prostaglandin biosynthesis on the carcinogen-induced development of mammary lesions in vitro. *Anticancer Res*, 11:587-592, 1991.
46. Gerhauser C, Woongchon M, Lee SK, Suh N, Luo-Kosmeder J, Luyengi J et al. Retinoids mediate potent cancer chemopreventive activity through transcriptional regulation of ornithine decarboxylase. *Nature Medicine*, 1:260-266, 1996.
47. Mehta RG, Hawthorne ME, Steele VE. Induction and prevention of carcinogen-induced precancerous lesions in mouse mammary gland organ culture. *Methods in Cell Science*:19:19-24, 1997.
48. Mukherjee R, Daview PJ, Crombie DL, Bishoff ED, Cesario RM, Jow L, et al. Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature*, (386)407-410, 1997.
49. Kubota T, Koshizuka K, Williamson EA, Asou H, Said J, Holden S, et al. Ligand for Peroxisome proliferator-activated receptor  $\gamma$  (Troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. *Can*

- Res, 58:3344-3352, 1999.
50. Gottardis MM, Bischof E, Shirley MA, Wagoner MA, Lamph WWL, Heyman RA. Chemoprevention of mammary carcinoma by LGD1069 (Targretin): an RXR-selective ligand. *Canc Res*, 56:5566-5570, 1996.



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